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14. ABSTRACT
Insulin receptor (IR) and insulin-like growth factor 1 receptor (IGF1R) have been shown to have a role in breast cancer, although the specific importance of hybrid IR/IGF1R (Hybrid-R)is unknown. I proposed to use an inducible dimerization system to study Hybrid-R, and then optimize an assay to detect Hybrid-R in breast tumor samples. I constructed chimeric IR and IGF1R plasmids with different dimerization domains and developed stable MCF10A clones expressing only chimeric IR or chimeric IGF1R allowing homodimerization. Homodimerizer AP20187 treatment induced activation of chimeric IGF1R and IR in a dose dependent manner and activated AKT and ERK. However, I encountered technical difficulties in establishing cells expressing both chimeric IR and chimeric IGF1R and was unable to induce specific Hybrid-R. To measure the IR/IGF1R hybrid receptor on paraffin embedded specimens, a proximity ligation assay (PKLA) was optimized using both anti-IR and anti-IGF1R antibodies. The presence of PLA signals was increased in cells had IR or IGF1R overexpression and decreased in cells with IGF1R knock down. The variability of signals was also observed in a tissue microarray with normal breast tissue and breast tumor. This study shows the presence of Hybrid-R in breast cancer cell lines and human tumors and suggests that more studies are needed to determine its function and importance in breast cancer.

15. SUBJECT TERMS
Insulin Receptor (IR), IGF1R, IR/IGF1R Hybrid receptor (Hybrid-R), Proximity ligation assay (PLA), breast cancer

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1) INTRODUCTION

The Breast Center at Baylor College of Medicine (BCM) is an excellent teaching environment for graduate students. During my pre-doctoral training, I completed my coursework, passed my qualifying exam, and attended numerous lectures and courses on breast cancer. I presented my data on many occasions (listed in reportable outcomes). I felt very fortunate to receive such training. However, at the end of Year 2 of my training (December 2010), I became ill with an auto-immune disease and was forced to take a leave of absence (LOA) and return to my parents in Taiwan. My health didn't allow me to return to the USA to complete my studies and I had to finish my studies early with a M.Sc. (instead of the Ph.D.). The last year of my DOD pre-doctoral fellowship was thus not completed and funding was returned to the DOD. This final report (December 2011) is thus similar to the report in December 2010 when I took my LOA. The goal of my project was to better understand insulin receptor (IR) and insulin-like growth factor-1 receptor (IGF1R) action in breast cancer. IR mediates the endocrine actions of insulin to regulate glucose homeostasis and metabolism [1]. In contrast, the related insulin-like IGF1R signaling cascade is a major regulator of cell proliferation and survival. Both IR and IGF1R have been shown to have a role in many cancers including breast cancer [2-5]. IR/IGF1R hybrid receptors (Hybrid-R) have been shown to occur when IR and IGF1R are co-expressed[6]. Moreover, Hybrid-Rs are widely overexpressed in breast cancer specimens compared to normal breast tissue and the Hybrid-R levels are higher than both IR and IGF1R levels in most breast cancer specimens[7]. However, Hybrid-R signaling and role of Hybrid-R in breast cancer remain un-known due to the difficulty to study the Hybrid-R specifically since they co-exist with IR and IGF1R. **I hypothesized that Hybrid-Rs play a role in tumor progression by promoting cell proliferation, survival and migration, and that the levels of Hybrid-R may correlate with clinical outcome and biological characteristics of breast cancer patients.** In this study, I proposed to utilize an inducible dimerization system to identify signaling pathways driven by Hybrid-R and develop a new assay that allows us to measure Hybrid-R levels on paraffin embedded specimens then further evaluate the prognostic value of Hybrid-R in breast cancer.

2) BODY

In this project, we utilized an inducible dimerization system in collaboration with Dr. Muthuswamy from Cold Spring Harbor to generate chimeric IR and chimeric IGF1R to specifically study Hybrid-R. Chimeric IGF1R retroviral vector with FKBP domain (IGF1R-Fv2) and empty vectors with FKBP and FRB domain were provided by Dr. Muthuswamy. Homodimerizer AP20187 is used to dimerize chimeric IR with FKBP domain (IR-Fv2) or IGF1R-Fv2 and heterodimerizer AP21967 is used to dimerize IGF1R-Fv2 and chimeric IR with FRB (IR-FRB) to form Hybrid-R. The principle of inducible dimerization system for this project is attached in the Appendix I.

<u>Specific Aim 1:</u> Create breast cancer cell lines stably transfected with inducible receptors and identify signaling pathways and specific adaptor proteins activated by these inducible receptors.

Construct retroviral vectors and confirm the expression for chimeric IR and chimeric IGF1R protein expression

To construct IR-Fv2 that allows homodimerization, the beta sub-unit of IR was PCR amplified and then inserted into retroviral empty vector with FKBP domain using XbaI enzyme. The same strategy was used to create IR-FRB that allows heterodimerization with IGF1R-Fv2.

IGF1RFv2 vectors were transfected into retrovirus packaging cell line PT67 and cells were harvested after antibiotic selection for stable expression of chimeric IGF1R. As shown in Fig.1A, chimeric IGF1R proteins were detected using both IGF1R and HA antibodies and they have higher molecular weight than endogenous IGF1R proteins.

IR-Fv2 and IR-FRB vectors were transiently transfected into 293 cells and cells were harvested at 48 hours after transfection for immunoblot analysis. In Fig. 1B, the IR-Fv2 was detected using both IR and HA antibodies and the IR-FRB was detected using both IR and Glu-Glu antibodies. Both kind of chimeric IR ran at higher molecular weight than endogenous IR.

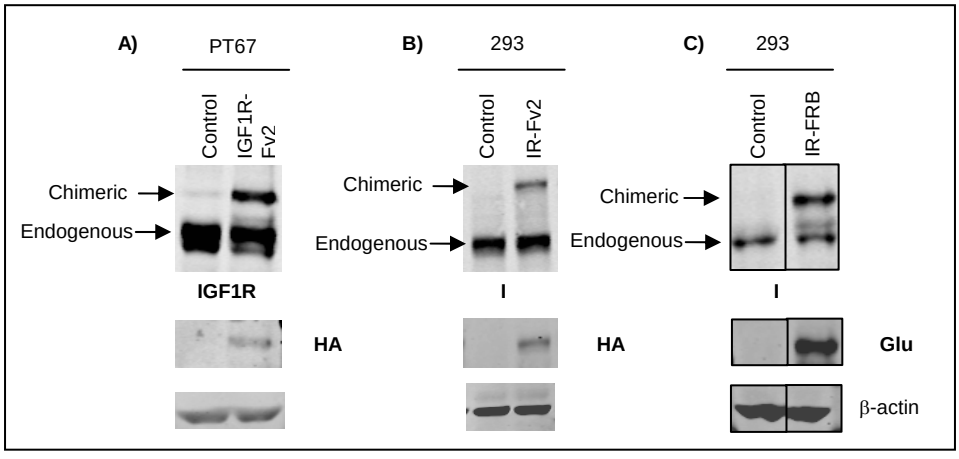

Figure 1. Expression of chimeric IR and IGF1R in transiently transfected 293 and PT67 cells. A) Transfected PT67 cells were harvested after antibiotic selection for stable expression of chimeric IGF1R. Proteins were harvested in 5% SDS buffer and analyzed by western blot for chimeric IGF1R expression using anti-IGF1R and anti-HA antibodies. B) and C) 293 cells were transiently transfected with chimeric IR with FKBP (IR-Fv2) or FRB domain (IR-FRB). Proteins were harvested at 48 hours after transfection for western blot analysis. Anti-IR and anti-HA antibodies were used to detect IR-Fv2 and anti-IR and anti-Glu-Glu antibodies were used to detect chimeric IR-FRB.

Establishing MCF10A cells expressing chimeric IR and/or chimeric IGF1R and their response to dimerizers

Retroviruses for IGF1R-Fv2 and IR-Fv2 were harvested from transient transfected PT67 cells for infection. Infected MCF10A cells were then subjected to 500ug/ml Geneticin selection. Geneticin resistant clones were harvested for immunoblot to confirm the IGF1R-Fv2 or IR-Fv2 expression. I have generated two stable MCF10A pools: IGF1R-Fv2 and IR-Fv2 by pooling all positive stable clones after screening. Prior to establishing stable MCF10A clones for IR-Fv2, I performed immunoblot analysis on MCF10A IGF1R-Fv2 pools treated with or without homodimerizer AP20187 to verify the functionality of IGF1R-Fv2 (Fig. 2). As expected, treatment of AP20187 successfully induced phosphorylation of chimeric IGF1R and activation of AKT and ERK. Interestingly, phosphorylation of IGF1R-Fv2 and AKT seemed to be saturated at the lowest dose (20nM) in this experiment. This suggested that a lower dose range should be used to further characterize the dose response of IR-Fv2 and IGF1R-Fv2.

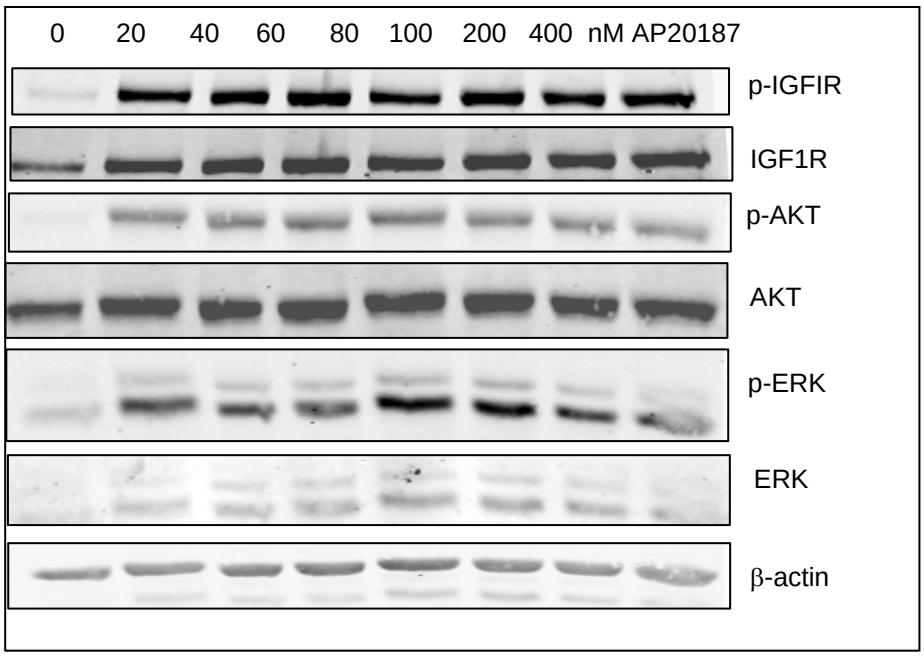

Figure 2. AP20187 induces activation of IGF1R-Fv2 and down stream AKT and ERK in MCF10A cells. MCF10A cells stably expressing IGF1R-Fv2 were treated with incremental doses of AP20187 for 15 minutes after serum free medium (SFM) starvation overnight. Proteins were detected by western blot using antibodies against p-IGFIR, IGF1R, p-AKT, AKT, p-ERK, ERK and β-actin.

After establishing stable clones for IR-Fv2, I used another dose range of AP20187 to test the functionality of IR-Fv2 in comparison of IGF1R-Fv2. We found that AP20187 was able to induce both IR-Fv2 and IGF1R-Fv2 activation and increase of AKT and ERK phosphorylation in a dose dependant manner (Fig 3). However, the magnitude of chimeric receptor and AKT activation in MCF10A IR-FV2 pool was much smaller compared to IGF1R-Fv2 pool. From the HA blot that detect the levels of IR-Fv2 and IGF1R-Fv2, it seems like the expression levels of transgene was much less in IR-Fv2 pool compared to IGF1R-Fv2 pool. I performed an immunoblot to compare the HA levels in all IR-Fv2 and IGF1R-Fv2 individual clones and found that 3 out of 4 IR-Fv2 clones had much less levels of transgene expression compared to IGF1R-Fv2 clones (data not shown). Low levels of transgene expression could be one of the reasons that the same dose of AP20187 wasn't able to induce the same levels of response in IR-FV2 cells. In addition, stable IR-Fv2 cells had longer doubling time and were not as health as stable

IGF1R-Fv2 cells as they produced much more debris in culture medium. It was hard to rule out if it's because of position of the transgene incorporated into the genome or in the process of antibiotic selection that led to this difference between stable IR-Fv2 and IGF1R-Fv2 cells. Therefore, we will try to re-establish another set of stable clones for IR-Fv2.

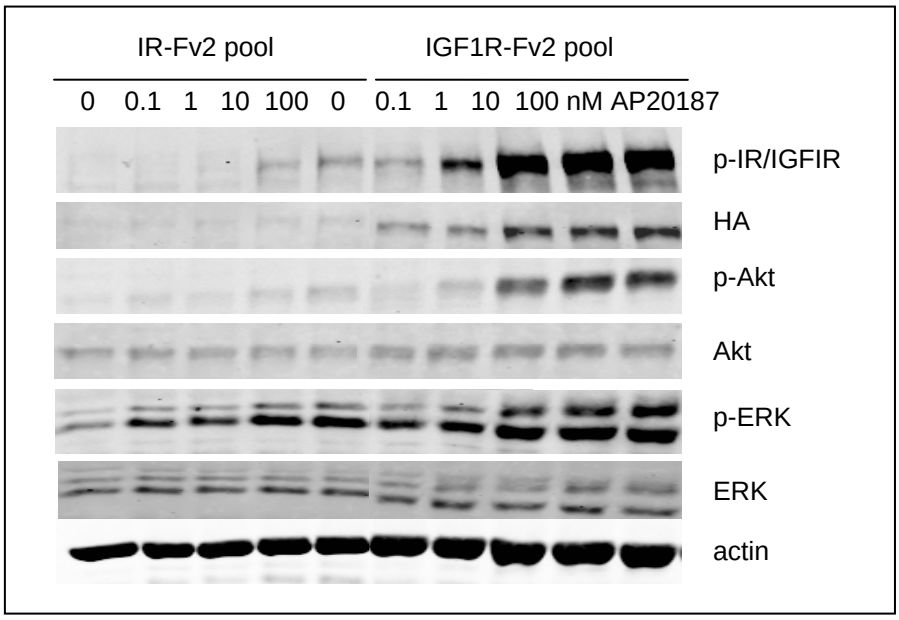

Figure 3. AP20187 induces activation of IR-Fv2 and IGF1R-Fv2 and down stream AKT and ERK in MCF10A cells. MCF10A IR-FV2 pool and IGF1R-Fv2 pool were generated by pooling all positive clones with 1 to 1 ratio. Cells were starved in SFM overnight then treated with incremental doses of AP20187 for 15 minutes. Protein levels were detected by western blot using antibodies against p-IR/IGF1R, HA, p-Akt, Akt, p-ERK, ERK and β-actin.

We also established three stable MCF10A clones expressing IR-FRB (data not shown). In order to create cells expressing both IR-FRB and IGF1R-Fv2 that allow us to induce IR/IGF1R Hybrid formation specifically, we had transfected them with IGF1R-Fv2. Transfected cells were put under both 500ug/ml Geneticin and 5ug/ml Puromycin selection to select for double positive cells. However, we had tried two sets of transfection and weren't able to establish stable clones in the process of clone expansion.

<u>Specific Aim 2:</u> Investigate phenotypic changes in breast cancer cells expressing IR/IGF1R hybrid receptor and identify signaling pathways that lead to these phenotypic changes.

We proposed to study if activation of IR/IGF1R Hybrid could promote cell proliferation or migration in MCF10A cells. However, we weren't able to establish stable cell lines expression both IGF1R-Fv2 and IR-FRB allowing us to continue the study. Since IR/IGF1R hybrid binds to both insulin and IGF, we felt it important to understand how different breast cell lines respond to insulin and IGF stimulation. Therefore, we initiated a comparative study for insulin and IGF action using a panel of breast cancer cell lines with endogenous IR, IGF-IR and Hybrd-R. In this study, we compared gene expression levels of insulin and IGF systems as well as multiplex proteomic profiling using reverse phase protein array (RPPA) to compare the characteristics and the relationship to insulin and IGF response in different breast cancer cell lines. From this study, we gained a better understanding of insulin and IGF signaling. The progress of this study will be listed after aim 3.

<u>Specific Aim 3:</u> Determine the levels of IR/IGF1R hybrid receptors in breast cancer samples and correlate these to clinical characteristics and outcome.

In the proposal, I proposed to use a FRET/FLIM technique to measure the Hybrid-R on paraffin embedded samples. However, I encountered some difficulties to find the microscope with FLIM capability and it is more difficult to quantify the loss of signal instead of gain of signal. Therefore, I decided to switch to proximity ligation assay (PLA) developed by Olink Bioscience to measure the Hybrid-R. The principle of PLA assay is attached in the Appendix II.

Create control samples for PLA assay development

I generated cell pellet controls using transient transfection of IR or IGF1R in 293 cells and siRNA knock down IGF1R in MDA-MB-134 to create samples with different levels of Hybrid-R expression. Transfected cells were harvested on parallel for cell lysate and paraffin embedded blocks. From immunoblot and immunohistochemistry (IHC) of IGF1R (Fig. 4A-C), we observed a significant decrease of signal on IGF1R siRNA transfected MDA-MB-134 cells and increase of signal on IGF1R transfected 293T cells. In addition, increase of IR expression was also confirmed in IR transfect 293T cells (Fig. 4C-D).

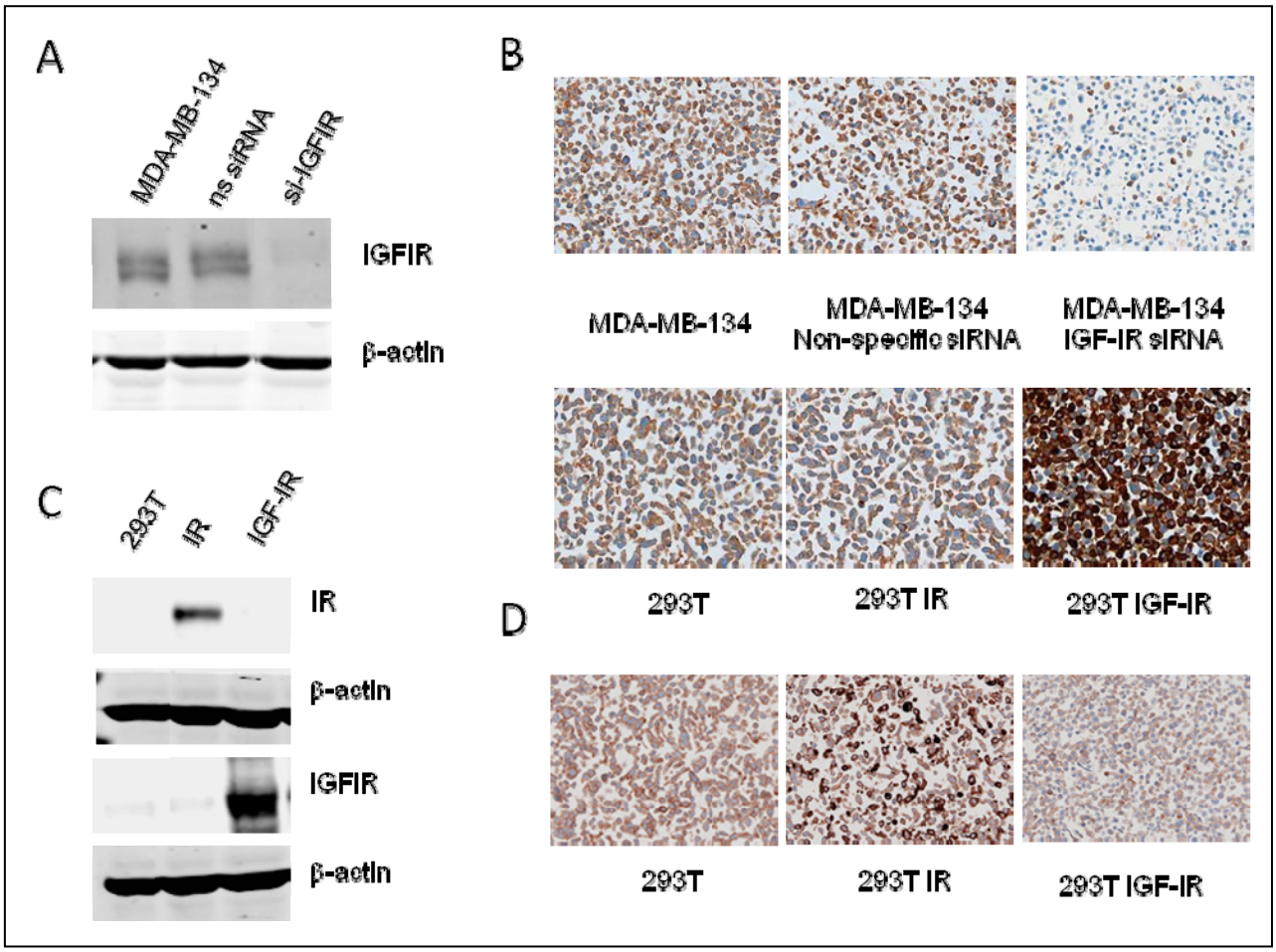

Figure 4. Immunoblot and IHC for IR and IGF1R in transfected MDA-MB-134 and 293T cells. MDA-MB-134 cells were transiently transfected with non-specific siRNA or IGF1R siRNA. 293T cells were transiently transfected with IR or IGF1R. Cells were scraped down and lysed in 5% SDS for immunoblot or fixed in 10% formalin for two hours prior embedding into paraffin block. Protein levels were detected by western blot using antibodies against IGF1R, IR and β-actin. A. Decrease of IGF1R expression in IGF1R siRNA transfected MDA-MB-134 cells. B. Immunoblot confirmed the increase of

IR and IGF1R expression in IR and IGF1R transfected 293T cells respectively. C. IGF1R IHC on MDA-MB-134 and 293T cell pellets. D. IR IHC on 293T cell pellets.

Given the significant investment of time and effort in developing positive controls for IGF1R staining, we went on to stain IGF1R levels by IHC in patient samples from a Phase 2 clinical trial of an anti-IGF1R antibody (Figitumumab, Pfizer) in non-small lung cancer. We found that IGF-IR levels were associated with response to Figitumumab and these were reported in Clinical Cancer Research (see reportable outcomes).

Optimization of PLA assay for Hybrid-R detection

To test if an antibody pair (IGF1R and IR) could be used for Hybrid-R detection in PLA assay, I used sections from MDA-MB-134 cell pellets to perform the PLA assay. In this experiment, I included several technical negative controls to confirm that the signal is truly from the dual recognition of IR and IGF1R antibody to the Hybrid-R, not from non-specific binding of probes or IR antibody or IGF1R antibody alone. As shown in figure 5, there was no signal detected on no antibody control, no IR antibody and no IGF1R antibody control. In contrast, there were bright red spots (indicated by white arrow in fig. 5 detected on MDA-MB-134 sections while the signal was absent in MDA-MB-134 section with IGF1R siRNA knock down.

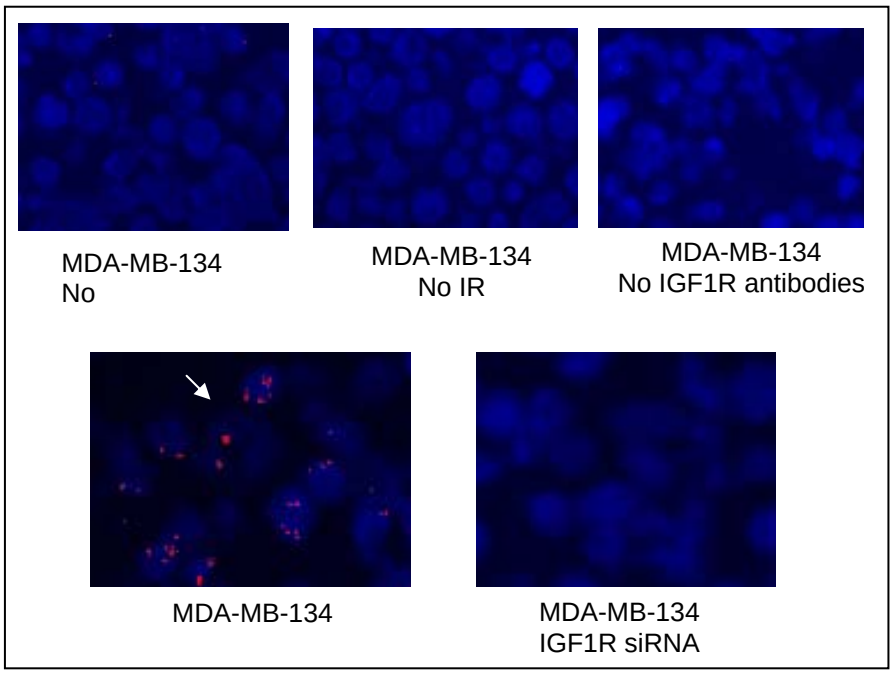

Figure 5. Detection of Hybrid-R on MDA-MB-134 cell pellet samples using PLA assay. MDA-MB-134 cell pellet sections were deparaffinized in Xylene. They were then placed in sodium citrate buffer and cooked for 20 minutes in pressure cooker for antigen retrieval. Blocking buffer provided in the PLA assay kit was used for blocking for 30 minutes. Monoclonal anti-IR antibody from Santa Cruz and Polyclonal anti-IGF1R antibody from Cell Signaling were used as primary antibody pair for duo recognition of Hybrid-R. The rest of the processes were performed according to the instruction from the PLA assay manual. The images were taken by Nuance system, a multispectral image system developed by CRI

I then performed another PLA assay on tissue microarray (TMA) that contains transfected cell pellet samples with different levels of IR or IGF1R expression. In accord with the observation from the last experiment, the signal was decreased in IGF1R siRNA transfected compared to non-specific siRNA transfected MDA-MB-134 cells. In addition, there was

increased of signals in IR or IGF1R transfected 293T cells compared to non-transfected control. (Fig. 6)

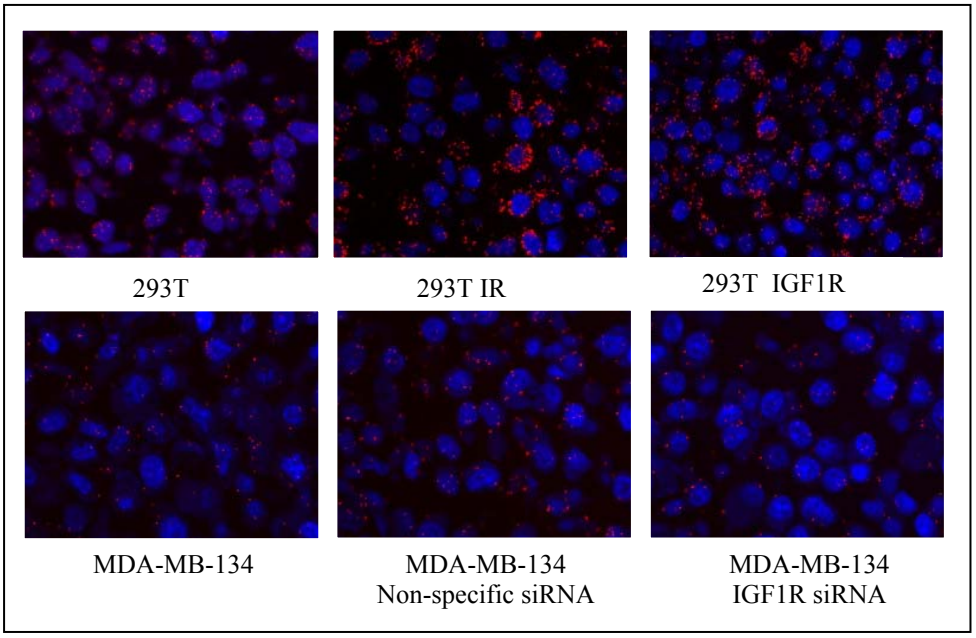

Figure 6. Difference of IR/ IGF1R Hybrid signals detected by PLA in transiently transfected 293T and MDA-MB-134 cells. Cell pellets with different levels of IR and IGF1R expression were made into a tissue microarray and 5μm sections were made for staining. Sections were deparaffinized and subjected to antigen retrieval as previously described. The blocking and primary antibody condition in previously experiment was followed and the rest of the processes were performed according to the instruction from the PLA assay manual. The images were taken by Nuance system, a multispectral image system developed by CRI

Next, I performed a PLA assay on a TMA slides with normal breast tissue and dead-end breast tumors to determine if the condition for cell pellet controls could also work on tissue samples. As shown in figure 7, the signal (indicated as white arrow) was present in some but not all tissue samples. However, it was difficult to visualize and quantify the staining signals in tissue samples due to the high fluorescence background.

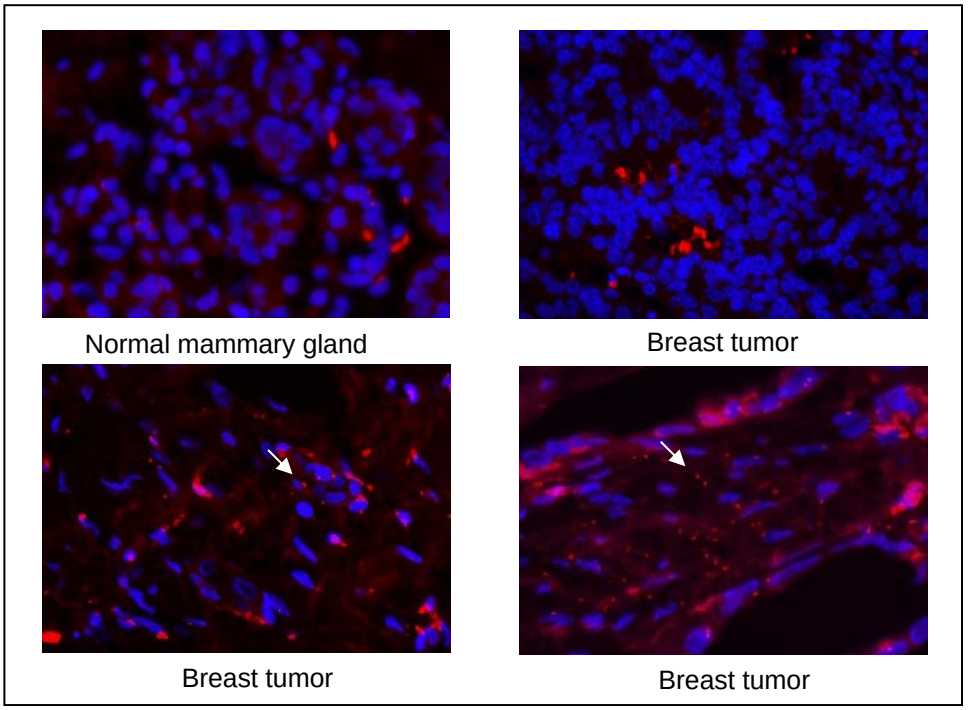

Figure 7. Detection dead end breast tissue samples using PLA assay. A TMA

slide with dead-end normal breast tissue and breast tumor specimens from Breast Center pathology core was used to test the PLA assay on tissue specimens. The staining condition used for cell pellet samples was followed in this assay and the images were also acquired using Nuance system.

I encountered some technical difficulties in reducing the fluorescence background in this fluorescence based PLA assay. Therefore, I also tried to the newly developed HRP-based PLA assay that would allow us to exam the staining result under bright field microscope, however, I couldn't produce reproducible results with this assay. The final result was that I was able to detect hybrid-R, and future studies will be to examine levels in a larger cohort of breast cancer patients.

Comparative transcriptional and proteomic profiling of insulin and IGF action in a large panel of breast cancer cell lines.

Breast cancer is a very heterogeneous disease and could be divided into subtypes based on clinical and molecular characteristics of tumors. Molecular analysis have been conducted on a collection of breast cancer cell lines in comparison with breast tumors suggesting that they could be used as models to study distinct subtypes of breast cancer [8]. To investigate if there is a potential correlation of insulin and IGF activities to specific breast cancer subtype, we decided to perform a serious of gene expression and protein profiling analysis in a panel of 21 breast cancer cell lines in our laboratory. Gene expression profile data from Neve *et. al*, 2006 was used in clustering analysis to compare insulin and IGF axis gene between different subtypes of breast cancer cell lines [8]. Hierarchical clustering analysis on combination of ligands, receptors, IRSs and IGFBPs probe subsets was performed and Basal B cell lines and PR positive cell lines were identified as distinct clusters. As shown in figure 8, luminal cell lines seem to be associated with higher insulin and IGF-I and IGF-II compared to basal A and basal B cell lines. Basal B cell lines seem to associate with higher IRS2 as well as IGFBP6 and IGFBP7 expression. In addition, analysis on subsets of probes was performed and ER positive cell lines were associated with higher IGFBP2, 4 and 5 (data not shown).

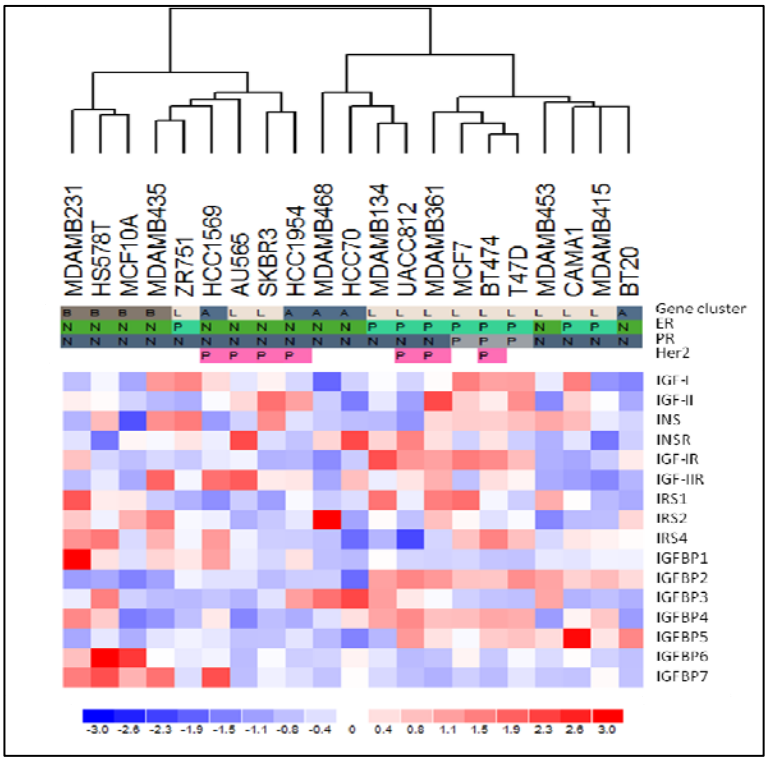

Figure 8. Insulin and IGF axis expression profile of 21 human breast cancer cell lines. Gene expression data from Neve et, al 2006 was used to compare markers insulin and IGF axis expression. Hierarchical clustering analysis was performed using probes for IGF-I, IGF-I, INS, IR, IGF1R, IGF-IIR, IRS1-4 and IGFBP1-7 on dCHIP software. Gene expression levels are presented by log2 pseudo-color scale.

We then measured IR and IGF1R mRNA as well as protein levels in the same panel of breast cancer cell lines and compare that to the gene expression analysis result. As shown in figure 9, IGF1R and IR receptor levels are very variable and no obvious association between IR and IGF1R level to specific subtypes of breast cancer cells was observed. However, high IR levels in MDA-MB-468 and ZR-75-1 and high IGF1R levels in MCF7 and MDA-MB-134 were generally in accord with the finding from Q-RT-PCR and RPPA (Figure 9).

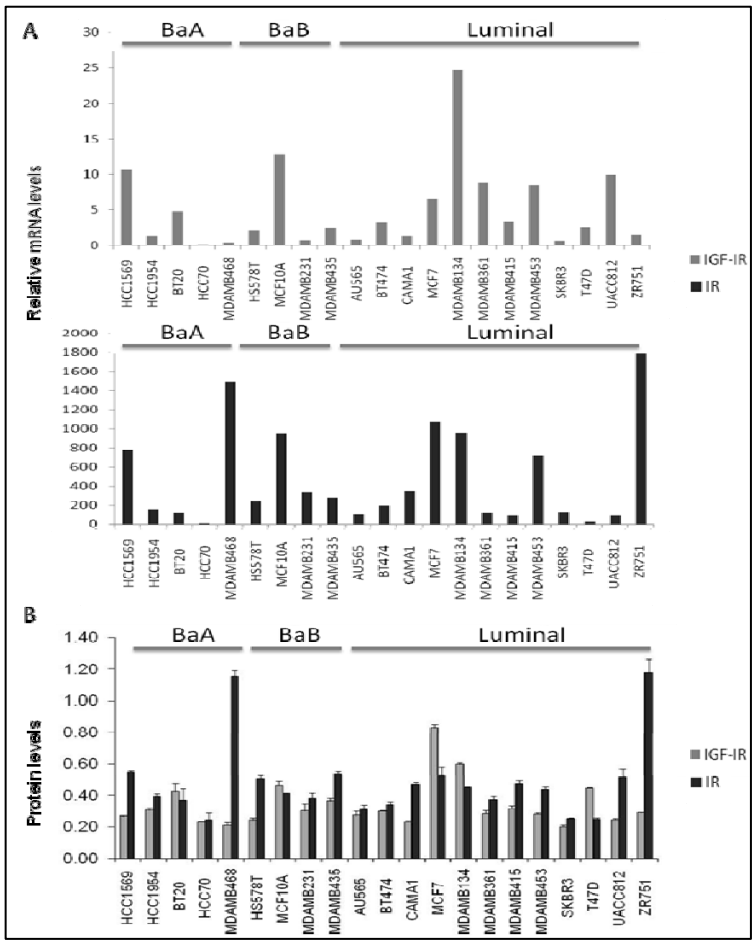

Figure 9. IR and IGF1R mRNA and protein in breast cancer cell lines measured by Q-RT-PCR and RPPA. A. Cell lines under normal culture condition were harvested and 1ug of mRNA were extracted and converted to cDNA via RT reaction. IR measured by SYBR and IGF1R by Taqman qPCR on 5ul of cDNA. B-actin was measured as control for gene expression normalization and ΔΔCT method relative quantification was used for comparison of relative fold of IR and IGF1R expression in different cell lines. Cell lines were arranged by gene clustered subtypes based on their transcriptional profile. B. Cells were cultured in triplicate were harvested in RPPA lysis buffer. IR and IGF1R protein levels were measured by RPPA and values from non treated control to compare receptor levels in this panel of breast cancer cell lines. Cell lines were arranged by gene clustered subtypes based on their transcriptional profile.

In order to gain better understanding the action of insulin and IGF in breast cancer, our lab utilized Reverse Phase Protein Array (RPPA) to study insulin and IGF induced protein profile in 21 different breast cancer cell lines in collaboration with Department of Systems Biology in MD Anderson. In this study, we stimulated 21 breast cancer cell lines with either insulin or IGF at 10nM for six different time points (5, 10, 30 minutes, 6, 24 and 48 hours) in triplicate and profiled 134 different phospho-protein and non-phospho protein markers that involved in growth factor signaling pathways as well as cell cycle regulation and DNA repair.

In order to identify effects induced by insulin and IGF, we used serum free non-treated control as baseline to determine the fold change of protein levels induced by insulin or IGF. In preliminary analysis, we selected protein changed for greater than 1.3 or -1.3 fold to generate insulin and IGF profile for each cell line and use dCHIP software for clustering analysis to compare them among different cell lines. As shown in figure 10, insulin and IGF induced similar protein profile within each cell line. However, there were differences of profile patterns within and between cell lines. Among these 21 cell lines, we identified several basal B

(MCF10A, MDA-MB-231 and MDA-MB-435) and luminal (MCF7, ZR-75-1, T47D, MDA-MB-134, and MDA-MB-415 and MDA-MB-453) cell lines with increase of markers activation in PI3K pathway upon IGF or insulin stimulation.

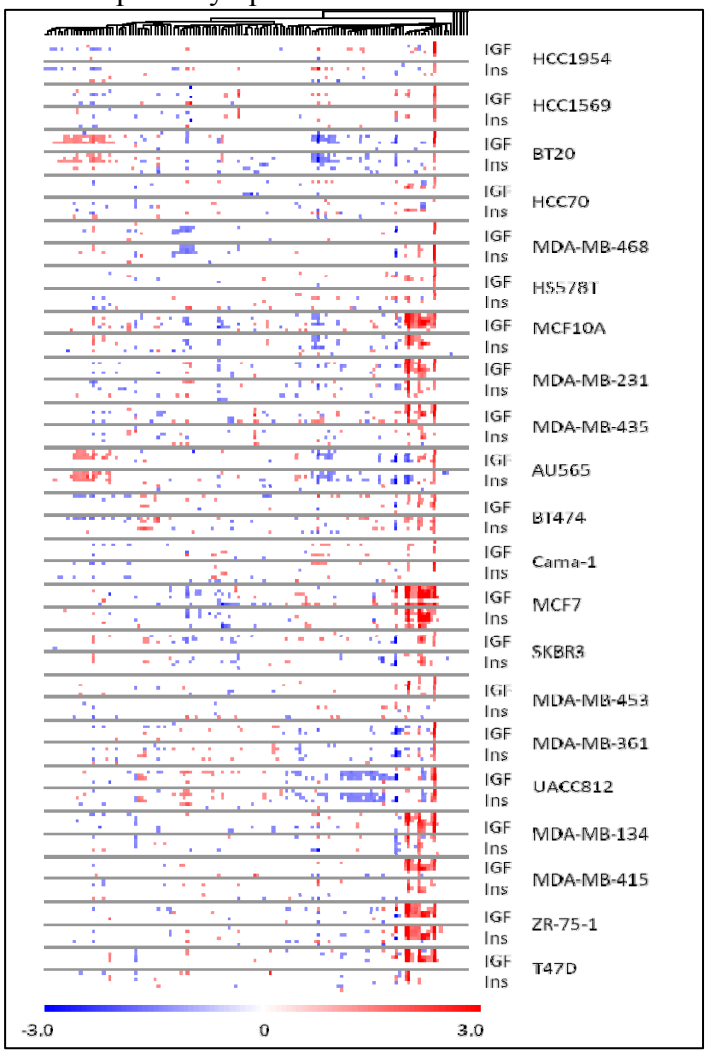

Figure 10. Clustering of insulin and IGF induced protein changes in 21 breast cancer cell lines. Thumbnail heatmap of clustering analysis of protein fold change induced by insulin and IGF throughout 6 different time points in 21 cell lines. Values from triplicate were averaged and serum free non treated controls were used as baseline for normalization and for relative protein fold change calculation. One-way ANOVA with contrast was performed to identify proteins that are statistically significantly modulated by insulin and IGF. In this analysis, protein fold changes greater than 1.3 fold (in red), less than -1.3 fold (in blue) were included in hierarchical analysis and depicted by log 2 pseudo-color scale shown (fold changes between 1.3 and -1.3 or non statistical significant were shown in blank). Protein fold changes within IGF or Insulin (Ins) row for each time point were presented in longitudinal order: 5, 10, 30 minutes, 6, 24 and 48 hours.

From this analysis, we found that majority of proteins with significant changes disappear at 24 and 48 hour time points and patterns from 10 minutes to 6 hour time points are generally consistent. We then performed hierarchical clustering analysis on results from 30 minutes time point and to identify cell lines with similar insulin and IGF response. As shown in figure 11, we found 2 groups of cells with distinct pattern. One group of cell lines clustered with MCF7 including both basal B and luminal cells and there seems to be no obvious correlation between ER status and the response to insulin or IGF. In this cluster, insulin or IGF treatment significantly induced AKT, S6K, p70S6K and STAT5 phosphorylation. Moreover, results of insulin and IGF treatment clustered tightly together in MCF7, MCF10A and ZR-75-1 cells. We then took a closer look at the insulin and IGF induced fold changes in these cell lines and found that IGF is more potent activator compared to insulin. For example, IGF induced more than 10 fold of AKT phosphorylation in contrast to 5 and 3 fold activation induced by insulin in MCF7 and MCF10A cells. However, ZR-75-1 cells which have significant high IR expression, insulin and IGF induced comparable fold AKT and MAPK phosphorylation. In addition, we found a group of cell lines had significant decrease of p-MAPK upon insulin or IGF stimulation including AU565, BT-20, UACC812, MDA-MB361, SKBR3, BT474 and MDA-MB-468. Interesting, majority of them

are Her2 positive cell lines except MDA-MB-468 which has EGFR overexpression suggesting insulin and IGF may play role in negative feedback mechanism in cell lines with other growth factor pathway activation. In the future, we are interested in comparing the effect of insulin and IGF in cell proliferation in cell lines that clustered with MCF7 and identifying mechanism for insulin and IGF induced MAPK de-phosphorylation in Her2 positive cell lines.

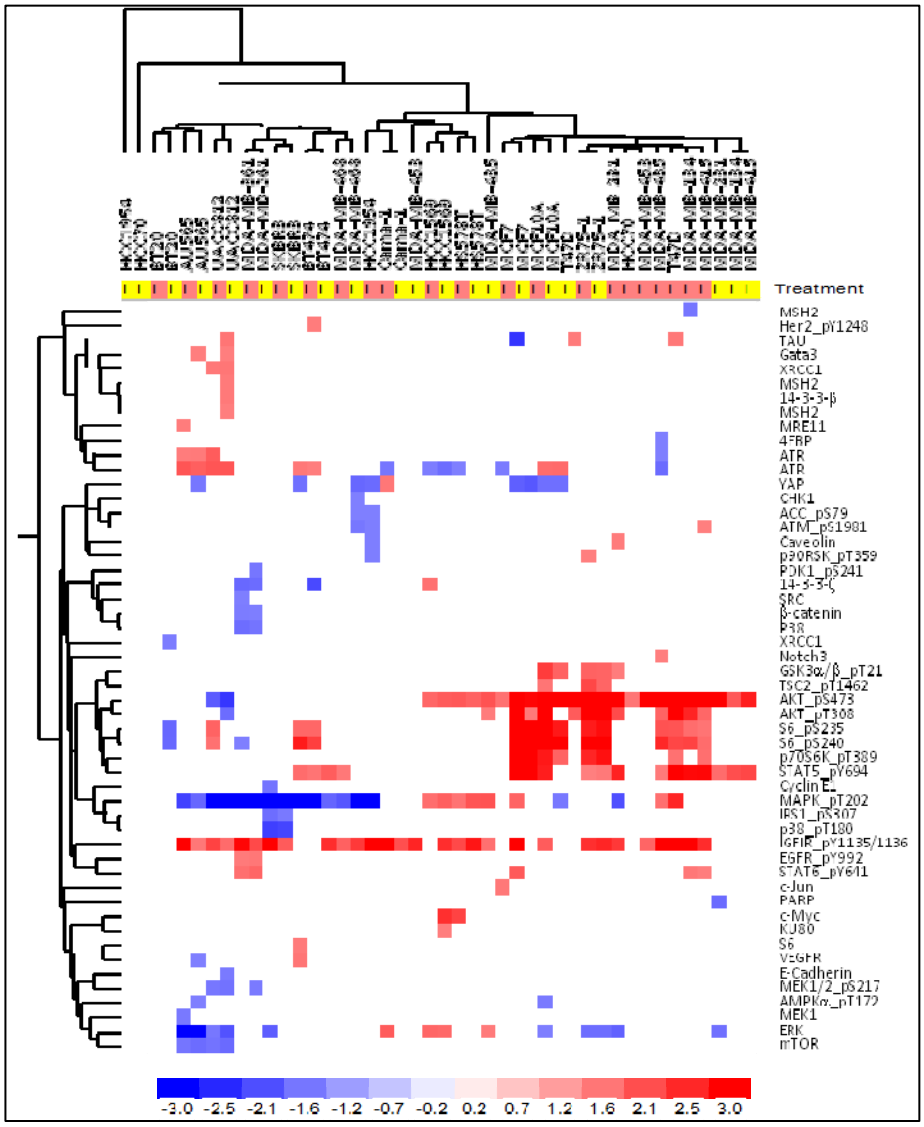

Figure 11. Clustering analysis of insulin and IGF protein identified 2 groups of cell lines with distinct response. Two way hierarchical clustering analysis of protein changed induced by 30 minutes of insulin or IGF treatment was perfomed. One-way ANOVA with contrast was performed to identify proteins that are statistically significantly modulated by insulin and IGF. In this analysis, protein fold changes greater than 1.5 fold (in red), less than -1.5 fold (in blue) were included in hierarchical analysis and depicted by log 2 pseudo-color scale shown (fold changes between 1.5 and -1.5 or non statistical significant were shown in blank). Insulin treament was coded in yellow and IGF treatment was coded in pick in dendrogram annotation.

In conclusion, we were able to characterize IR and IGF1R levels and activation in a panel of breast cancer cell lines to identify cell lines that are representative of patterns of response. This data is being finished up by another postdoctoral fellow and will be used to continue preclinical studies of IGF1R and IR inhibitors in breast cancer.

3) KEY RESEARCH ACCOMPLISHMENTS

- Confirmed the expression and functionality of IGF1R-Fv2 in MCF10A cells.
- Constructed retroviral vector for IR-Fv2 and IR-FRB and confirmed the expression of chimeric IR with FKBP and chimeric IR with FRB in transiently transfected 293 cells.

- Established stable MCF10A clones for IR-Fv2 and IR-FRB.
- Optimized PLA assay for IR/IGF1R Hybrid receptor detection in paraffin embedded specimens.
- Measured IGF-IR in a phase 2 trial of Figitumumab in NSCLC and found that IGF-IR levels correlated with response.
- Identified Hybrid-R in breast cancer cell lines and human tissues.
- Preliminary analysis of insulin and IGF induced protein changes in 21 breast cancer cell lines showed different patterns of response.

4) REPORTABLE OUTCOMES

A) Manuscripts

1) Gualberto A, Dolled-Filhart M, Gustavson M, Christiansen J, **Wang YF**, Hixon ML, Reynolds J, McDonald S, Ang A, Rimm DL, Langer CJ, Blakely J, Garland L, Paz-Ares LG, Karp DD, Lee AV. Molecular analysis of non-small cell lung cancer identifies subsets with different sensitivity to insulin-like growth factor I receptor inhibition. Clin Cancer Res. 2010 Sep 15;16(18):4654-65.

2) Dearth RK, Kuiatse I, **Wang YF**, Liao L, Hilsenbeck SG, Brown PH, Xu J, Lee AV. A moderate elevation of circulating levels of IGF-I does not alter ErbB2 induced mammary tumorigenesis. BMC Cancer. 2011 Aug 25;11:377. PMID: 21867536

B) Presentations

1) Attended and presented data in poster format at San Antonio Breast Cancer Symposium (SABC) in San Antonio, TX, 2009

2) Attended American Association of Cancer Research Annual Meeting (AACR) in Washington D.C., 2010

3) Attended and presented data in poster format at the BCM Translational Biology and Molecular Medicine (TBMM) Research Conference at Houston Marriott Hotel, Houston, TX, 2010

4) Oral presentation at TBMM research seminar in Houston, TX, 2010

5) Attended and presented data in poster format at Graduate School Symposium, at BCM, Houston, TX, 2010

C) Degrees

MSc, June 2011

D) Employment

Professional Sales Representative (Oncology) at Glaxo Smithkiline (promoting therapeutic agents for Breast, Ovarian, Cervical cancer, SCLC, and ITP)

5) CONCLUSIONS

It has been very difficult to specifically study the role of IR/IGF1R Hybrid receptor (Hybrid-R) since it is impossible to stimulate endogenous Hybrid-R without activating IR or IGF1R. Therefore we proposed to utilize inducible dimerization system to differentiate the effect of Hybrid-R from that of IR and IGF1R. Unfortunately, we experienced technical difficulties in creating stable IR-Fv2 clones and double positive clones for IR-FRB + IGFIR-Fv2 in immortalized epithelial cell lines MCF10A. As working on inducible chimeric receptor

system, we realized that more studies are needed to compare the insulin and IGF responses in different breast cancer cells. Therefore, we embarked on a study to compare the gene expression profile and insulin and IGF induced protein changes in a panel 21 breast cancer cell lines. From this study, we gained insights into patterns of cell line response to IGF and insulin which will direct future preclinical studies. For example, IGF1R activation is known to be important in many aspects of tumor progression and therapeutic agents are developed to target IGF1R. There are 2 main categories of IGF1R therapeutic agents: anti-IGF1R antibody that is specific to IGF1R and tyrosine kinase inhibitor (TKI) that inhibit both IR and IGF1R. Oral administer of TKI is easier than infusion of monoclonal antibody into patients but inhibit insulin signaling will be more toxic to patients. However, insulin signaling activation could be one resistant mechanism to anti-IGFIR antibody therapy due to the high homology of these two pathways. From the preliminary analysis of the RPPA result we have identified cell lines are responsive to both insulin or IGF stimulation and they could further be used as candidate cell lines to study insulin action and their relationship to anti-IGF1R therapy.

The objective of developing the PLA assay was to allow us to have a better way to measure and quantify IR/IGF1R Hybrid in archived breast tumor samples that are mostly in paraffin embedded format and determine if levels of IR/IGF1R hybrid could have any prognostic significance for breast cancer. The preliminary result of PLA assay on cell pellet samples was promising and we observed difference of signal in a set of breast tissue samples. However, the high fluorescence background makes it technically challenging to quantify the signals. An initial attempt at optimizing an HRP-based PLA assay was not successful. Future plans are thus to use the fluorescence based assay on a large cohort of breast cancers to assess effects of the Hybrid-R on breast cancer prognosis.

In summary, I have received an outstanding training from Dr Lee (my mentor), the Breast Center at BCM, and my DOD pre-doctoral fellowship. This resulted in two co-authored publications, and hopefully more to come as my work is continued by others in Dr Lee's laboratory. I presented my work on many occasions and feel that my work is an important stepping stone for future studies. I was fortunate to received my MSc and now currently work for Glaxo Smithkline advancing drugs in breast cancer.

6) REFERENCES

1. Dupont, J. and D. LeRoith, *Insulin and insulin-like growth factor I receptors: similarities and differences in signal transduction.* Horm Res, 2001. **55 Suppl 2**: p. 22-6.
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5. Samani, A.A., et al., *The role of the IGF system in cancer growth and metastasis: overview and recent insights.* Endocr Rev, 2007. **28**(1): p. 20-47.
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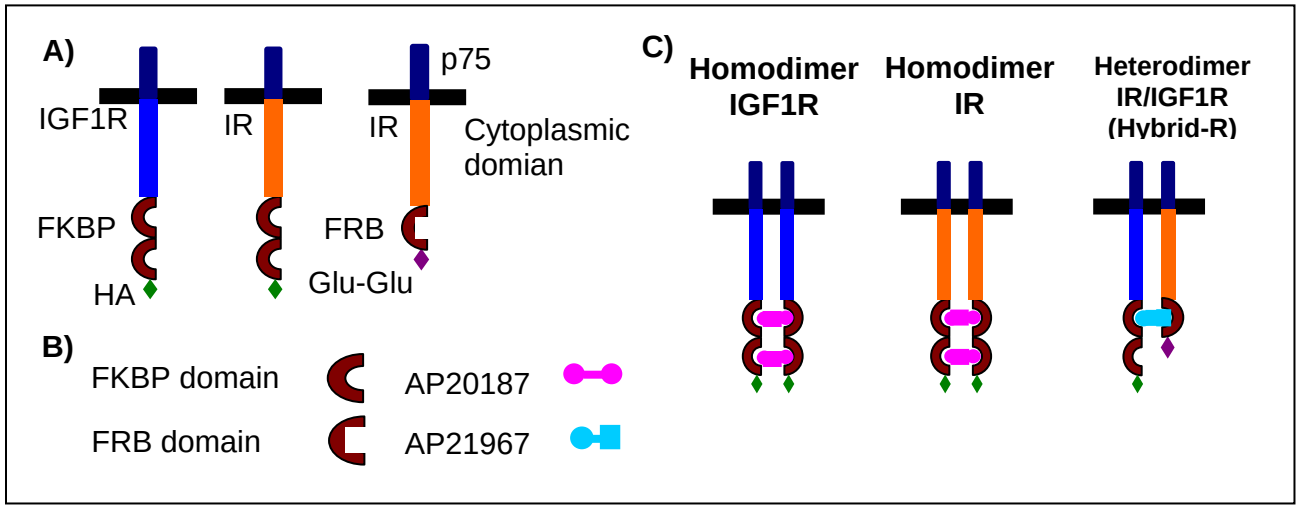
8. Neve, R.M., et al., *A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes.* Cancer Cell, 2006. **10**(6): p. 515-27.

7) **APPENDIX**

I. Inducible dimerization system



Each chimeric receptor consists of extra-cellular domain of p75 that avoids the ligand binding (insulin, IGF-I and IGF-II) to the chimeric receptor, beta sub-unit of IR or IGF1R, dimerization domain (FKBP or FRB) and a tag (HA or Glu-Glu). There are three different chimeric receptors in this system: (1) Chimeric IGF1R with FKBP domain (IGF1R-Fv2) tagged with HA (2) Chimeric IR with FKBP domain (IR-Fv2) tagged with HA (3) Chimeric IR with FRB domain (IR-FRB) was tagged with Glu-Glu. There are two kinds of dimerizers: AP20187 that brings two FKBP domains together for homodimerization and AP21967 that brings one FKBP and one FRB domain together for heterodimerization.

II. Principle of PLA assay (from Duolink PLA manual by Olink Bioscience)

Typical starting materials are adherent cells, cytospin preparations or tissue sections on a glass slide, fixed, pre-treated and blocked with a blocking reagent according to the requirements of the primary antibodies used.

1: The samples are incubated with primary antibodies that bind to the protein(s) to be detected.

2: Secondary antibodies conjugated with oligonucleotides (PLA probe MINUS and PLA probe PLUS) are added to the reaction and incubated.

3: The Hybridization solution, consisting of two oligonucleotides (illustrated as red bands), is added and the oligonucleotides will hybridize to the two PLA probes if they are in close proximity.

4: The Ligation solution is added together with Ligase (yellow), joining the two hybridized oligonucleotides to a closed circle.

5: The Amplification solution, consisting of nucleotides (not shown) is added together with Polymerase (yellow). The oligonucleotide arm of one of the PLA probes acts as a primer for a rolling-circle amplification (RCA) reaction using the ligated circle as a template, generating a concatemeric (repeated sequence) product extending from the oligonucleotide arm of the PLA probe.

6: The Detection solution, consisting of fluorescently labeled oligonucleotides, is added and the labeled oligonucleotides will hybridize to the RCA product. The signal is easily visible as a distinct fluorescent dot and analyzed by fluorescence microscopy.